

Activity Evaluation of Leaves' Extracts of
Funtumia Africana Stapf (Apocynaceae)
towards Methicillin-Resistant
Staphylococcus Aureus (MRSA) and
Phytochemical ScreeningKouadio NJ^{1,2,4}, BG Kipre^{1,2,4*}, Guessennd NK^{1,3}, Kouassi KA^{2,4}, Yao K^{2,4} and Kone MW^{1,2,4}¹Département de Bactériologie/Virologie, Institut Pasteur de Côte d'Ivoire (IPCI), Côte d'Ivoire²Université Nangui Abrogoua (UNA), Côte d'Ivoire³Université Félix Houphouët Boigny (UFHB), Côte d'Ivoire⁴Centre Suisse de Recherches Scientifiques en Côte d'Ivoire. Côte d'Ivoire

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Abstract

Funtumia africana Stapf. (Apocynaceae) is among the diverse medicinal plants of Ivorian National flora used for several pathologies' treatment. Complexity in curing some of the diseases associated with bacteria resistance, brought up inefficiency of a great number of current antimicrobial drugs. The present study evaluates the antibacterial activity of the crude extracts of the leaves of *F. africana* against methicillin-resistant *Staphylococcus aureus*. Additionally, secondary metabolites were screened out to discover new groups of molecular compounds responsible for specific activity. Bioassay experiments used were disk diffusion and Muller-Hinton broth dilution methods, to respectively determine the inhibition zone diameters and antibacterial key factors. As for the phytochemical investigation, test tubes colorimetric method was performed. Results showed that hydro-alcoholic and aqueous extracts of *F. africana* displayed strong activity with respective inhibition zone diameter comprised between 17.4 mm and 25.7 mm, and 16.7 mm and 19.7 mm. For both types of extracts, the Minimum Inhibition Concentration (MIC) varied from 0.046 to 1.56 mg/mL, demonstrating as a result, a bactericidal effect against the majority of strains tested. The current work, therefore justifies the use of *F. africana* as traditional medicine against bacterial infections.

Introduction

In developing countries, infectious diseases constitute a public health concern for being recurrent and deadly [1]. To illustrate, half of the 17 millions of deaths due to them, was recorded in Africa [2].

For this reason, antibiotic drugs discovery was a relief for humanity because it allowed to significantly deplete the incidence of infectious diseases, especially in western countries [3]. Nevertheless, the inappropriate use of antimicrobials brought about bacteria multidrug-resistance. Among these pathogenic agents, are *Staphylococcus aureus*. In developing countries, for cultural and financial reasons, people address plant-based medications to treat infectious diseases. The great interest for traditional medicine could be explained by the higher cost of modern medication added up to the emergence of bacteria resistance [4]. In order to face these obstacles to public healthcare, urgent initiatives were taken to seek for natural compounds potentially active and efficient for patients' effective treatment. To achieve this goal, medicinal plants were explored as an immense reservoir. That encompasses antibacterial bearing species. In so doing, *Funtumia africana* Stapf (Apocynaceae) was identified as a traditional medication that cures pneumonia, women infertility, coughing, diarrhea, urinary infections, tuberculosis and chronic wounds [5]. Therefore, the present work aims at evaluating the antibacterial activity of the leaf extracts of *F. africana* against methicillin-resistant *Staphylococcus aureus*. In addition, secondary metabolites were screened out to reveal prospective new active compounds.

Materials and Methods

Materials

The plant materials were essentially comprised of extracts obtained from leaves' powder of *Funtumia africana*. The leaves were collected in Lakota, in the western region of Côte d'Ivoire and located at a distance 244 km from Abidjan. They were then identified at the herbarium of Centre National de Floristique, Université Félix Houphouët Boigny in Côte d'Ivoire, and registered under voucher number 15838.

Table 1: List of bacteria strains studied.

Bacteria	Biological Products	Phenotype
1541C/10	Urines	MRSA,CRQF
1000C/14	Dental Cary	MRSA,CRQF
446C/14	Urines	MRSA,CRQF
499C/14	Peritoneal Liquid	MRSA,CRQF, MLST06
485C/14	Urines	MRSA,CRQF, MLSbi, KTG
408C/14	Urines	MRSA,CRQF, KTG, MLbc+S
ATCC 25923		Reference (Susceptible)

ATCC: American Type Culture Collection, MRSA: Methicillin-Resistant *Staphylococcus Aureus*, CRQF: Cross Resistance to Quinolone Fluoride, MLST06: Phenotype M (by efflux pump), MLSbi: Phenotype M (by efflux pump), MLbc+S: Phenotype M (by efflux pump), KTG: Kanamycin, Tobramycin, Gentamycin.

The bacteria set was made up of seven strains, with six clinical isolates of *Staphylococcus aureus* resistant to methicillin and one reference material as outlined in Table 1. Apart from biological material, solvents, culture media and routine laboratory equipment were used.

Methods

Crude extracts preparation: Crude extracts were obtained using method performed in previous experimental study [6]. Three solvents (hexane, methanol 70% and distilled water) were used to carry out the extraction process, which could be described as follows: 125 g of plant powder were macerated in 1 L of hexane for 24 h. Then the filtrate obtained went through evaporation using a rotor vapor, and dried under fume board to generate hexane extracts. The remaining marc residue was dried on filter paper and weighted. Afterwards, it was macerated in 1L of methanol 70% for 24 h. Then, the filtrate was evaporated off to give an aqueous marc, which was incubated at 50°C to obtain methanol 70% extracts. The aqueous extract was also prepared by maceration of 125 g of plant powder using 1L of distilled water as solvent, and filtrate was dried at 50°C in an incubator.

Bioassays

Bacteria inoculum preparation for Muller-Hinton Agar diffusion: Bacteria inoculum was prepared from a fresh colony of 18 to 24 h, which was homogenized in 2 mL suspension of NaCl 85%. Then, optical density was adjusted to 0.5 McFarland. Thereafter, 1000 µL of the suspension obtained was dispensed in 10 mL of saline solvent (NaCl 0.9%).

Efficacy test: The test was carried out using Muller Hinton Agar disk diffusion method [7]. This consisted of preparing 100 mg/mL of extract by dissolving 500 mg of plant powder in 5 mL of sterile distilled water. Then, Petri dishes were streak-seeded with the inoculum, followed by making 3 mm diameter wells, which were filled in with 50 µL of the extract sample and this set was incubated at 37°C for 24 h. Finally, reading of inhibition zone diameter was done, and results were presented as Mean ± SD (**Figure**) because the experiment was performed in triplicate. Afterwards, data analysis was performed using GraphPad Prism 5 software (One-way ANOVA and Turkey Multiple Comparison test) to assess activity difference of the plant extracts. Plant extract efficacy was evaluated according to the

Ponce et al. criteria [8]. Thus, a strain is said to be resistant to an antimicrobial agent when the inhibition diameter is lesser than 8 mm, whereas susceptibility is detected when this diameter is comprised between 9 and 14 mm. In case it falls within 15 and 19 mm, the strain is declared highly susceptible, and above 20 mm it is said extremely susceptible to the antimicrobial drug extremely susceptible to the antimicrobial drug.

Bacteria inoculum preparation for broth dilution method: Twenty four hours colonies were collected using a pasteurized pipette, and then dispensed in a 10 mL test tube containing Muller-Hinton sterile broth. The mixture was incubated at 37°C for 3 h. After that, 0.3 mL of the seeds obtained were diluted into 10 mL of Muller-Hinton sterile broth and stirred up to get a suspension.

Serial dilution of the initial plant concentration: Initial plant extract concentration was serially diluted with distilled water from 50 mg/mL to 0.092 mg/mL.

Determination of antimicrobial key parameters

The antimicrobial parameters were evaluated using broth dilution method [9]. Thus, 1mL of known concentration of plant extract and 1 mL bacteria inoculum were added up to a series of 10 test tubes. Each individual test tube was vortexed to get a good reaction mixture. Then, the growth control test tube was filled in with 1mL of distilled water and 1 mL of inoculum, whereas the sterility control tube contained the sterile Muller-Hinton broth. The final concentration in the test tubes varied from 25 to 0.046 mg/mL. These tubes were incubated at 37°C for 24 h. The Minimum Inhibitory Concentration (MIC) is the lowest plant extract concentration for which no bacteria growth was observed. While the minimum bactericidal concentration is the one that allows observation of at least 0.01% of viable bacteria. To achieve this goal, initial bacteria inoculum was diluted from 10⁻¹ to 10⁻⁴, and streak-seeded 5 cm long using a 2 µL calibrated loop on a Muller-Hinton Agar gel prior to incubation of Petri dishes at 37°C for 24 h. These dishes were labeled A. After reading the MIC values, the tube content where no bacteria growth was observed, was streak-seeded 5 cm long using a 2 µL calibrated loop on Muller-Hinton Agar gel. The latter dishes were labeled B. Then, by comparison of dish A and dish B, the minimum bactericidal concentration was determined. Finally, the MBC/MIC ratio defined the activity trend criteria. As a matter of fact, when MBC/MIC is lower or equal to 2, the test compound is said bactericidal, and bacteriostatic in otherwise case.

Phytochemical screening of the plant extracts

Polyphenols: Iron chloride test (FeCl₃): To 2 mL of plant extract, a drop of alcoholic solution of Iron chloride 2% is added up, and Phenols are revealed as a result of a blue-black or green color more or less darkened.

Catechic tannins: Stiasny test: catechic tannins are revealed revealed using Stiasny reagent. 5 mL of extract solution are evaporated off, followed by addition of 10 mL mixture of 75 mL formaldehyde 30% and 25 mL of concentrated hydrochloric acid to the marc residue. The new mixture obtained is subjected to water bath (80°C) for 30 min and cooled off. The presence of catechic tannins is ascertained by brownish flocculation.

Gallic tannins: Sodium acetate test: Revelation of gallic tannins is obtained by saturation of the filtrate that came out of the catechic

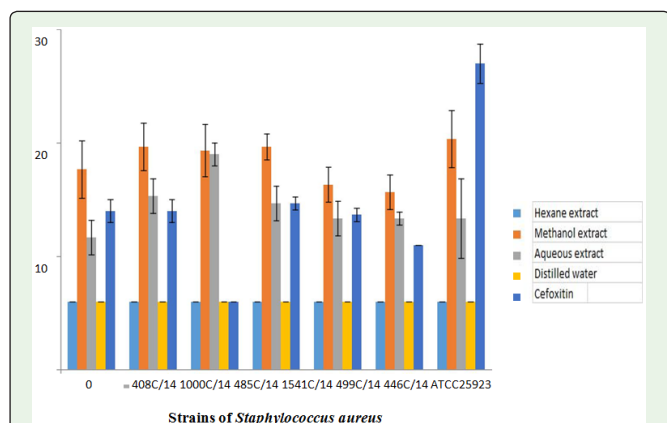


Figure.1: vertical axis (inhibition zone diameter) of extracts of *Funtumia africana* (Mean ± SD) for triplicate experiments on *Staphylococcus aureus*.

tannins and addition of a drop of alcoholic ferrous chloride 2%. Gallic tannins are revealed as a result of an intense blue-black color.

Anthocyanins: Hydrochloric acid test: Anthocyanins are revealed by hydrochloric acid reaction upon the plant extract. A 2 mL of prospective tested compound solution is mixed up with 2 mL hydrochloric acid (2 N), and a pink-reddish color appears. Then, by addition of ammonia, this color turned into blue-purple, bringing about the presence of anthocyanins.

Flavonoids: Sodium hydroxide test: One to two drops of NaOH 10% is added up to 1 mL plant extract, and an intense yellow color is observed marking the presence of flavonoids.

Alkaloids: Dragendorff test: The presence of alkaloids is revealed by use of Dragendorff reagent. To achieve this result, the solvent of extract solution is evaporated off, and then the remaining residue mixed with 6 mL of methanol before adding up one or two drops of Dragendorff reagent. The red-orangish precipitate indicates the presence of alkaloids.

Free anthraquinones: After solvent evaporation of extract solution, 2 mL of distilled water is added up. To this mixture is then added 4 mL of chloroform, and by use of a separatory funnel, both the organic phase and aqueous phase are obtained. In the final step, 0.5 mL of ammonia 10% is added up, and the presence of free anthraquinones is indicated by pinkish color of the aqueous phase.

Heteroside anthraquinones: Solvent evaporation of the plant extract solution was carried out using sand heating. After this step, 5 mL HCl

10% is mixed with the extract residue. This mixture is submitted to a water bath for 30 min. After cooling off, the hydrolysate is mixed with 10 mL of chloroform in a separatory funnel. Then, the non-organic phase is half diluted in 0.5 mL of aqueous ammonia, and the presence of anthraquinones with sugar moieties is indicated by a red color.

Saponins: Saponins are detected by the foaming formation test, which consists of plant extract solvent evaporation (2 mL) prior to adding up 5 mL of distilled water. Afterwards, the test tubes are vigorously shaken, and 1 cm height foaming indicates the presence of saponins.

Sterols and polyterpens: Salkowski test: Sterols and polyterpens are revealed using Salkowski reaction. To a 1 mL of each extract solution tested, two drops of concentrated sulfuric acid are added up and a yellow or red color indicates the presence of sterols and polyterpens.

Results

Antibacterial activity

The hydro-alcoholic and aqueous extracts showed an inhibitory activity against strains studied. The inhibition zone diameter varied from 17.4 mm to 25.7 mm for methanol 70% extract, and from 16.7 mm to 19.7 mm for aqueous extract (Figure 1). Moreover, the methanol 70% displayed stronger efficacy towards strain 1541C/14 with inhibition diameter of 25.7 mm, while aqueous extract showed significant activity on strain 408C/14 with inhibition zone diameter of 19.7 mm. At reverse, hexane extracts showed no activity against the strains. Assessing the antibacterial key factors, bacteria growth was depleted in a reverse relationship with extract concentration in the test tube. The outcome of this experiment indicated MIC values comprised within 0.046 and 0.92 mg/mL for methanol 70% extract. Whereas, for the aqueous extract they fall within 0.185 to 0.78 mg/mL. As for the MBC, methanol 70% showed values that varied from 0.092 to 0.185 mg/mL, while the aqueous extract gave values that ranged from 0.37 to 3.125 mg/mL (Table 2). Furthermore, methanol 70% extract of *Funtumia Africana* was bactericidal against 100% of the bacteria tested, when the aqueous extract showed the same trend towards 57% of strains studied.

Natural compounds revealed by phytochemical screening

The phytochemical screening carried out upon crude extracts of the leaves of *Funtumia Africana* indicated the presence of different molecular compounds (Table 3) made up of saponins, catechic tannins, flavonoids, polyterpens and polyphenols. Nevertheless, they did not contain anthocyanins. Finally, it could not be left unmentioned that hexane extracts did not show presence of compounds sought for.

Table 2: Activity evaluation of *Funtumia Africana* on Methicillin-resistant *S.aureus*.

Strains	Methanol 70% extract				Aqueous extract			
	MIC	MBC	MBC	Effect	MIC	MBC	MBC	Effect
			MIC				MIC	
408C/14	0.046	0.092	2	Bcidal	0.185	0.37	2	Bcidal
1000C/14	0.092	0.185	2	Bcidal	0.78	1.56	2	Bcidal
485C/14	0.046	0.092	2	Bcidal	0.78	1.56	2	Bcidal
1541C/14	0.046	0.092	2	Bcidal	0.78	3.125	4	Btic
499C/14	0.046	0.092	2	Bcidal	0.37	1.56	4	Btic
446C/14	0.046	0.092	2	Bcidal	0.78	3.125	4	Btic
ATCC25923	0.046	0.092	2	Bcidal	0.78	1.56	2	Bcidal

Bcidal: Bactericidal; Btic: Bacteriostatic; MBC: Minimum Bactericidal Concentration(mg/mL); MIC: Minimum Inhibitory Concentration (mg/mL).

Table 3: Chemical compounds screened out from the leaves of *Funtumia Africana*.

Chemical compound	Extracts		
	Hexane	Methanol 70%	Distilled water
Saponins	--	+	+
Catechic tannins	--	+	+
Gallic tannins	--	--	--
Alkaloids	--	--	--
Flavonoids	--	+	+
Anthocyanins	--	--	--
Polyterpens	--	+	+
Polyphenols	--	+	+
Heterosides anthraquinones	--	--	--
--: Absence ; +: Presence			

Discussion

The present study aimed at evaluating the antibacterial activity of the hexane, methanol 70% and aqueous extracts of leaves of *Funtumia Africana* against the *in vitro* growth of methicillin-resistant *S.aureus* strains. Results from this investigation showed strong efficacy of the named extracts against bacteria studied with bactericidal effect respectively upon 100% of the strains for hydro-alcoholic extract and 57% for aqueous extract. This property of plant extracts could surely justify its use in traditional medicine to cure pathogenic diseases like boil, whitlow, diarrhea, urinary infections, chronic wounds, especially Buruli ulcer for which *S.aureus* is the pathogenic agent of surinfection [10]. Aside this, known chemical compounds bearing antibacterial activity [11] were screened out (Table 3). They comprised tannins, flavonoids, heteroside anthraquinones and polyterpens. This result is another reason of the use of the plant part as traditional medication [12]. As for hexane extracts, which did not show presence of any molecular compound, they could be either non-soluble or slightly soluble in this solvent. Though, Methanol 70% and aqueous leaf extracts from *Funtumia Africana* showed an inhibitory activity against *in vitro* bacteria growth. In addition, methanol 70% extract was highly active than aqueous extract. But, antibacterial activity showed by both extracts could explain a prospective synergistic effect of molecular compounds extracted by methanol 70% and distilled water [13]. The methanol 70% extract of the plant had a bactericidal effect against bacteria strains studied with MIC values within 0.046 and 0.092 mg/mL. These results correlate with work done earlier on [14] in South Africa, whereby antibacterial activity of the leaves of *Funtumia Africana* against methicillin-resistant *S.aureus* was demonstrated. On the other side, comparison of this previous study with the current work showed higher inhibition zone diameters displayed by the latter investigation. The increased diameter values could be due to the different methods used. For, instead of using filter paper disk soaked with plant extract [7], 3 mm wells were made at equal distance in the Muller-Hinton Agar gel, where crude extract of different concentrations were dispensed for efficient diffusion [9]. Difference in inhibition zone diameter could also be explained by plant leaves collection either at day light, or at sunset as carried out in the present case. This, because active principles are more concentrated in plant parts from sunset to midnight [15]. Additionally, when

comparing this study with work done by Chuah EL. et al. [16], the methanol leaf extract of *Funtumia Africana* showed stronger activity against MRSA (Table 2) than methanol leaf extracts of *Bauhinia purpurea* (5.000 mg/mL), *Dicranopteris linearis* (0.625 mg/mL), *Melastoma malabathricum* (0.156 mg/mL), except *M. calabura* (0.039 mg/mL). These four South East Asian plants are respectively from Leguminosae, Gleicheniaceae, Melastomatacea and Elaeocarpacea families. Another comparison with early studies by B.G. Kipre, et al. [17], displayed MIC values of *F. Africana* to be of great interest.

Conclusion

To sum up, the present study showed the antibacterial activity of the leaves of *Funtumia Africana* against strains of methicillin-resistant *S.aureus*. It also allowed the evaluation of MIC and MBC values; the latter proving that the activity was mostly bactericidal. From this perspective, the plant was competitive, and demonstrated strong potential to be an antibacterial agent towards MRSA. Additionally, molecular compound groups, probably responsible for the named activity, were revealed and their presence was significant in hydro-alcoholic solvent. Provided these crucial results, further investigations are being performed to allow a best use of the plant. That is, in depth purification of the plant extracts and bio-guided molecular compounds separation for structure elucidation.

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